REMARKS

Entry of the foregoing, reexamination and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested.

By the foregoing amendment, claims 4, 5, 15, 21, 22, 23 and 26 have been amended. In particular, claims 4, 5, 21, 22, and 23 have been amended to recite that , even though hydrogen peroxide is removed prior to subjecting the material to UV light, a residual or trace quantity of hydrogen peroxide absorbed by or located adjacent to any microorganisms present on the material is retained. Support for such amendments can be found throughout the originally filed application, including for instance page 7, lines 3-9 and page 11, lines 13-17. Claims 15 and 26 have been amended for grammatical-type purposes. No new matter has been added.

SUBSTANCE OF INTERVIEW

Applicant acknowledges the courtesy extended to the undersigned representative by Examiner Chorbaji and Primary Examiner Jastrzab during the interview conducted on August 4, 2004.

While all of the claims were presented during the interview, applicant's representative focused on the independent claims since the arguments traversing the rejections of such independent claims apply equally to the dependent claims.

In the interview, the anticipation rejection which utilizes the DiGeronimo patent, United States Patent No. 4,494,357 (hereinafter "the '357 patent"), was discussed. The obviousness rejections, which all utilize the '357 patent as the primary reference, were also discussed. The specific arguments presented by applicant's representative during the interview with regard to the anticipation

rejection and the obviousness rejections are incorporated below in applicant's response to each individual rejection.

RECTION UNDER 35 U.S.C. § 102(b)

Claims 5, 17, 21, 23 and 25-26 have been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by the '357 patent. This rejection is respectfully traversed.

For prior art to be anticipatory, every element of the claimed invention must be disclosed in a single item of prior art in the form literally defined in the claim. *See, e.g., Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986). Here, the '357 patent fails to disclose **every element** of applicant's claimed invention **in the form literally defined in the claims**.

In the Official Action, the Examiner cites to the discussion of hydrogen peroxide treatment in column 4 (tables 2-3) of the '357 patent and figure 2 of the '357 patent for the drying and UV irradiation steps. However, contrary to the Examiner's conclusion, the '357 patent fails to teach an apparatus or method in which material is treated with hydrogen peroxide, a substantial amount of hydrogen peroxide is removed by applying air, and then the material is subjected to UV radiation.

The discussion of hydrogen peroxide treatment in columns 2-4 of the '357 patent do not use air treatment in combination with UV radiation. Rather, the use of hydrogen peroxide in columns 2-4 are either hydrogen peroxide treatment alone or hydrogen peroxide and heat. As can be seen from the '357 patent, the discussion of hydrogen peroxide in columns 2-4, was for purposes of comparing the effect of conventional techniques on microorganisms against the invention of the '357 patent which was directed to the combination of ultraviolet and ultrasonic irradiation. No

place in columns 2-4, or in any other location for that matter, does the '357 patent disclose hydrogen peroxide treatment, air **and** UV radiation.

Figure 2 of the '357 patent, which is the disclosed invention of the '357 patent, depicts a continuous sterilization process that begins with a liquid bath or vat in which ultrasonic energy would be radiated through the liquid, then air knives for drying the material and next a source of ultraviolet light. With regard to the ultrasonic energy, it is acknowledged that applicant's claims do not exclude such treatment. Nonetheless, the '357 patent fails to disclose, either explicitly or inherently, that the liquid in the bath can be or is hydrogen peroxide. The only mention in the '357 patent of what the liquid may be in the vat or bath of Figure 2 is located in claim 1 of the '357 patent. Claim 1 of the '357 patent recites that the apparatus includes "a vat of liquid free of chemical biocide . . . ." Hydrogen peroxide is not a liquid "free of chemical biocide."

Thus, every element of the present inventor's claimed invention, is not dislclosed in the '357 patent **in the form literally defined in the claims**.

Moreover, the '357 patent fails to disclose, either explicitly or inherently, that hydrogen peroxide is removed while retaining a residual or trace quantity of hydrogen peroxide which is absorbed by or located adjacent to any microorganisms present on the material. It is the synergy between this residual or trace quantity of hydrogen peroxide which is retained and the UV radiation which kills the microorganisms on the material. Thus, **every element** of applicant's claimed invention is not disclosed by the '357 patent

For at least the reasons discussed above, the '357 patent fails to disclose every element of applicant's apparatus and method claims in the form literally

defined in such claims. Accordingly, the '357 patent is not anticipatory prior art. Withdrawal of this rejection under 35 U.S.C. § 102(b) is thus respectfully traversed.

REJECTIONS UNDER 35 U.S.C. § 103(a)

The Examiner has set forth a number of rejections under 35 U.S.C. § 103(a) which all utilize the '357 patent as the sole or primary reference. Specifically, the rejections are as follows:

(i) claims 2-3, 15, 22 and 24 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over the '357 patent;

(ii) claim 4 has been rejected under 35 U.S.C. § 103(a) as being unpatentable over the '357 patent in view of Loliger et al. (United States Patent No. 3,692,468);

(iii) claim 6 has been rejected under 35 U.S.C. § 103(a) as being unpatentable over the '357 patent in view of Lagunas-Solare et al. (United States Patent No. 5,364,645);

(iv) claims 18 and 27 been rejected under 35 U.S.C. § 103(a) as being unpatentable over the '357 patent in view of Castberg et al. (United States Patent No. 5,744,094).

Applicant respectfully traverses each of these rejections as the '357 patent, taken alone or in combination with any of the other patents cited by the Examiner, fails to teach or suggest applicant's claimed methods and apparatuses

To establish a *prima facie* case of obviousness, the Examiner still must provide for every element of the claimed invention either through the combination of references or the reference(s) modified by the Examiner in view of the contemporary knowledge in the field at the time of the claimed invention.

The '357 patent does not teach or suggest every element of applicant's claimed methods and apparatuses. As discussed above, the '357 patent fails to teach an apparatus or method in which material is treated with hydrogen peroxide, a substantial amount of hydrogen peroxide is removed by applying air, and then the material is subjected to UV radiation. The '357 patent also fails to suggest an apparatus or method utilizing the combination of steps.

While Figure 2 depicts a continuous sterilization process which begins with a liquid bath or vat in which ultrasonic energy would be radiated through the liquid, then air knives for drying the material and next a source of ultraviolet light, the '357 patent teaches away from using a product like hydrogen peroxide as the liquid in the bath. Specifically, column 2, lines 19-23 state that an advantage of the sterilization process of the '357 patent (which includes Figure 2) is that sterilization can occur without use of any chemical sterilants which may have undesirable properties. Also, claim 1 of the '357 patent specifically recites that the vat of liquid is free of chemical biocide. Since the '357 patent teaches away from the present inventor's claimed invention, the '357 patent lacks the necessary motivation to utilize hydrogen peroxide in combination with the air knives and UV radiation. Thus, one of ordinary skill in the art would neither make the combination nor possess a reasonable expectation of success in doing so.

Additionally, while Figure 2 of the '357 patent provides a means for drying the sonicated material by air knives, the '357 patent makes no mention as to how much liquid may be removed and/or retained. As discussed previously, the important discovery made by the present inventor is that, even though hydrogen peroxide is removed from the packaging sheet material, a residual or trace quantity of the

hydrogen peroxide is absorbed or otherwise retained or adjacent to any microorganisms that are present on the packaging sheet material. This residual or trace quantity of hydrogen peroxide which is absorbed by or otherwise retained in or near the microorganisms is thus available to react synergistically with the UV light to render the microorganisms non-viable and/or sterilize the material.

Attached hereto as Exhibit A is a copy of an article by Reidmiller et al. (Journal of Food Protection, 66(7): 1233-40 (2003)). The Reidmiller et al. article provides results of the synergy between hydrogen peroxide that is retained or absorbed by microorganisms after drying and UV irradiation to kill microorganisms in sterilization procedures for packaging materials. See, for instance, Figure 5.

Neither the Loliger et al patent, the Lagunas-Solare et al. patent, nor the Castberg et al. patent remedy the serious deficiencies in the '357 patent. Thus, even if the Loliger et al patent, the Lagunas-Solare et al. patent, or the Castberg et al. patent were to be combined with the '357 patent, one of ordinary skill in the art would still not arrive at the present inventor's claimed methods and apparatuses.

Accordingly, a proper *prima facie* case of obviousness has not been established. Therefore, withdrawal of each of the obviousness rejections set forth in the Official Action is respectfully requested.

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this Reply, or the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney concerning such questions so that the prosecution of this application may be expedited.

Respectfully submitted,

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Date: August 23, 2004

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EXHIBIT A

Journal of Food Protection, Vol. 66, No. 7, 2003, Pages 1233–1240

Characterization of UV-Peroxide Killing of Bacterial Spores

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ABSTRACT

Advantage is taken in many sterilization processes, especially for food packaging materials, of the synergy between H_2O_2 and UV irradiation for spore killing. The nature of the synergy is currently not well defined in terms of targets and mechanisms. We found that under some experimental conditions, the synergistic killing of spores of *Bacillus megaterium* ATCC 19213 appeared to be mainly UV-enhanced peroxide killing, while under other conditions, it appeared to be mainly peroxide-enhanced UV killing. Lethal combinations of H_2O_2 and UV irradiation for spores resulted in only modest increases in auxotrophic mutations among survivors, indicative of little DNA damage, in contrast to higher mutation levels after dry-heat damage at 115°C. However, the combination of UV light and peroxide did lead to major inactivation of glucose 6-phosphate dehydrogenase, an enzyme that was used to monitor the damage to bacterial protein. Synergistic UV-H_2O_2 killing was reduced by agents such as pyruvate, thiosulfate, and iron or copper cations, which appeared to act at least in part by reacting chemically with H_2O_2, and was only slightly affected by the use of UV light at a wavelength of 222 nm rather than 254 nm. Hydrogen peroxide treatment can precede UV irradiation for synergistic killing by some hours with an interim of drying for spores of *Bacillus subtilis* A, a spore type used commonly for the validation of aseptic processes. Synergistic killing of dried spores or those in suspensions was accelerated at higher temperatures (50°C) rather than at lower temperatures (25°C).

UV irradiation and hydrogen peroxide can act synergistically to kill bacteria, both vegetative cells and spores. Results of initial studies by Bayliss and Waites *(2–4)* indicate that the two agents had to act at the same time rather than sequentially for synergy. Advantage has been taken of this synergistic action to design more effective sterilization procedures that have been especially directed to the killing of spores. Its major application has been seen in aseptic packaging and in the processing of packaging materials undergoing peroxide sterilization, which will subsequently be used with products sterilized by ultrahigh-temperature processes. Aseptic technology is now being applied to an increasingly wide variety of products from foods to pharmaceuticals. The optimal wavelength of irradiation that will kill spores in the presence of H_2O_2 is approximately 270 nm, with an effective range from about 240 to 290 nm *(16)*. The optimal H_2O_2 concentration for UV-H_2O_2 killing at 20°C was about 0.3 M (ca. 0.92%) for spores of *Bacillus subtilis (2),* and killing was reduced at higher concentrations, probably because of UV shielding by the peroxide. Use of 0.3 M of H_2O_2 alone was not lethal for the spores. The sporicidal action of H_2O_2 is very temperature sensitive *(14),* so that, at higher temperatures, much lower concentrations of H_2O_2, which shield less, are highly effective for killing. In essence, the shielding effect can be reduced and the sporicidal potency increased simply by raising the temperature.

Although Waites et al. *(16)* suggested that the mechanism for UV-H_2O_2 synergy is related to enhanced production of hydroxyl radicals from H_2O_2 due to irradiation rather than to any direct interactions of UV light with DNA, there remain many questions about the nature of killing by the combined agents. Is it mainly peroxide killing or UV killing, or is it somehow different from the two? What are the targets and mechanisms for killing? In this study, we have characterized spore killing by these combined agents in terms of induction of auxotrophic mutation, a sign of DNA damage, and in terms of the inactivation of a sentinel enzyme within intact spores, a sign of protein damage. We have also assessed the protective effects of transition metal cations, thiosulfate, dimethylthiourea, and pyruvate. In addition, we have investigated the feasibility of using a shorter wavelength of UV (222 nm) for spore killing and for augmenting peroxide damage.

MATERIALS AND METHODS

Spores. Spores of *Bacillus megaterium* ATCC 19213 and *Clostridium sporogenes* ATCC 7955 were prepared as described previously *(8)*. Spores of *B. subtilis* A (ATCC 9372 and NCA 7252) were prepared with the sporulation agar of Kim and Naylor *(7)*. Spores were purified from cultures after lysis of sporangia by repeated centrifugation and the scraping of pellets to remove vegetative debris. The final preparations consisted only of phase-bright spores as viewed under a phase-contrast microscope with a 90°-positive plate. They were stored under USP 200 proof ethanol in the cold until used.

Killing assays. Killing assays were carried out as described previously *(14)* with spore suspensions containing approximately 10^9 CFU per ml of 1% (wt/vol) (Difco, Detroit, Mich.) peptone broth. Subsequent dilutions were in 1% Difco peptone broth, and 0.1-ml samples were streak plated on Trypticase soy agar. The addition of catalase to the peptone dilution medium did not enhance survival nor did the addition of glucose to the plating me-

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dium or the use of Plate Count Agar (Difco) instead of Trypticase soy agar. Plates were incubated for at least 48 h at 37°C to allow the full development of colonies. UV-peroxide killing was somewhat reduced, by about 50% in terms of the rate of killing, for suspensions in peptone compared with those in water or salt solution, but the final levels of killing were unaffected. Also, the killing rate could be increased somewhat by lowering the initial population levels to 10^7 to 10^8 CFU per ml, possibly because of reduced shielding. However, we preferred to maintain high population levels to more closely approximate heavily contaminated suspensions. Also, when spores were dried on glass coverslips or filter paper strips, population densities were high, even when dilute suspensions were used. The important point is that in any set of experiments, conditions of suspending medium and spore density were kept constant to keep shielding at as constant a value as possible. In fact, it is impossible to have no shielding, even with dilute spore suspensions in water. Viable counts of spores of *C. sporogenes* were determined by means of the standard most probable number assay with three tubes per dilution. All experiments were repeated at least twice. Data are presented with error bars indicating standard deviations when the number of replicates of the same experiment was three or more.

UV irradiation at 254 nm was carried out with a Sterilaire Series unit (UVP Ultra-violet Products, Cambridge, UK) with two 15-W mercury tubes, which produce mainly UV radiation at 254 nm at an intensity of 1,600 $\mu W/cm^2$ at a distance of 30.5 cm. A UVX radiometer and a sensor calibrated for radiation at 254 nm were used routinely to assess output. UV irradiation at 222 nm was carried out with an L-Bluelight Excimer Module from Heraeus Noblelight GmbH (Hanau, Germany). For irradiation, 5 ml of spore suspension was placed in a glass petri dish with a diameter of 8 cm and an area of 50 cm^2. One-milliliter samples of the suspension were withdrawn at intervals during irradiation. For combined treatments, the spores were suspended at the time of irradiation in peroxide solutions. Most experiments were carried out at 25°C. The temperature was increased to as high as 50°C by use of a solid, thermostatted, heating block. For irradiation of dried spores, aliquots of suspensions were dried on standard glass coverslips (18 by 18 mm). For combined UV-H_2O_2 treatments, the peroxide was added to the spore suspension before drying. The results of previous studies *(12)* indicate that spores have a high capacity to retain peroxides during drying. UV doses were estimated by use of calibrated radiachromic strips (FWT-60-00; Far West Technology, Inc., Goleta, Calif.).

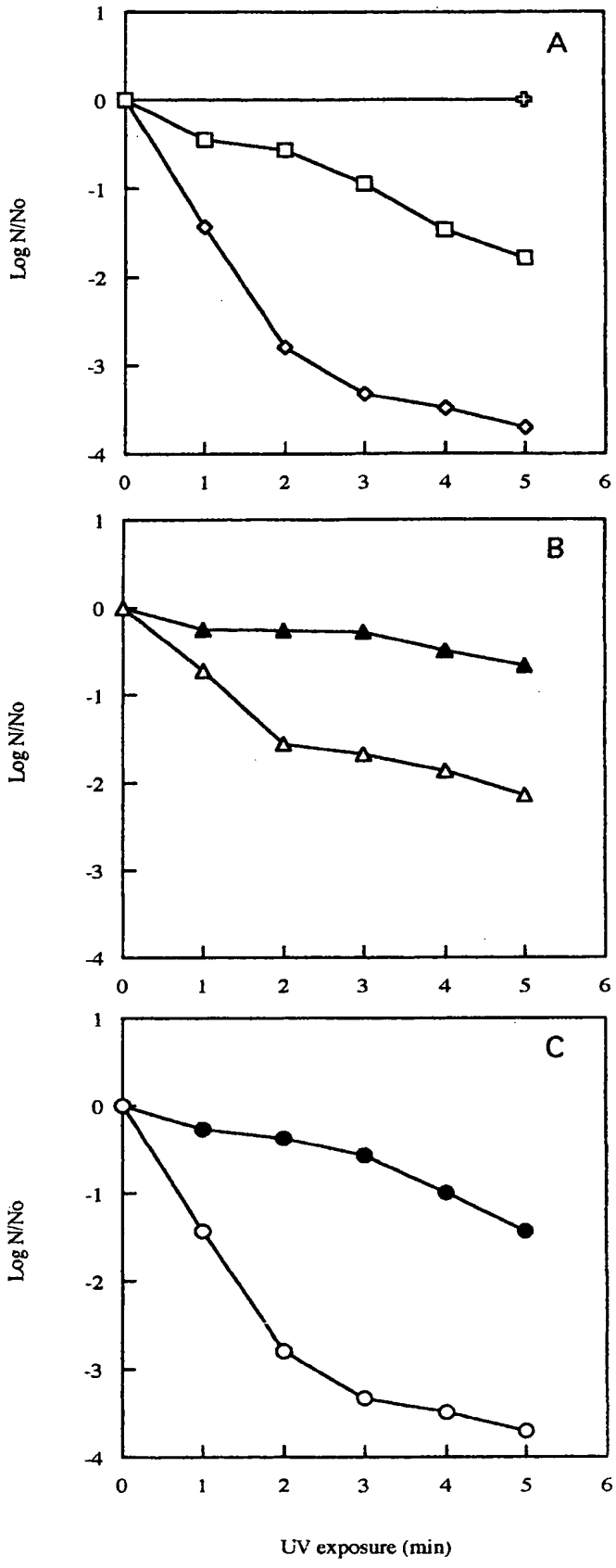
Mutation assays. Auxotrophic mutations were detected by replica plating survivors of UV irradiation, H_2O_2 treatment, or combination treatment on defined Slepecky-Foster medium and complete Trypticase soy agar medium, as described previously *(11)*. Spores treated with dry heat were used as positive controls for mutagenesis. All mutant colonies were subcultured on the two media to confirm that they were auxotrophic mutants able to grow on Trypticase soy but not on Slepecky-Foster agar. The levels of killing for inducing mutations ended with between 0.01 and 0.1% survival. Survivors were characterized from multiple experiments over a period of months, which allowed us the ability to test close to 2,000 colonies of survivors for UV, H_2O_2, or UV-H_2O_2 treatments and close to 1,000 for dry-heat treatment.

Determination of enzyme inactivation. Details of the assay of the enzyme glucose 6-phosphate dehydrogenase from intact spores of *B. megaterium* were given by Palop et al. *(11)*. This assay involves monitoring changes in the absorbance of light at 340 nm because of the reduction of NADP that results from the conversion of glucose 6-phosphate to 6-phosphogluconate. Basically, intact spores were exposed to the damaging agents and then washed and germinated in the presence of chloramphenicol to block protein synthesis. The germinated spores were then permeabilized with toluene and freezing before enzyme assays. The treatments had a tendency to slow germination but did not reduce the fraction of the spores becoming phase-dark when viewed microscopically in response to germinants. Moreover, some enzymes, such as F_1ATPases, were not affected *(11)* by the treatments and so could not serve as indicators of UV-peroxide damage to enzymes; however, they were good indicators that the germination and permeabilization procedures were effective for treated spores.

RESULTS

Factors affecting spore killing by UV irradiation and peroxides. The data in Figure 1A show the well-known synergistic interaction between H_2O_2 and UV irradiation for spore killing, in this case, for spores of *B. megaterium* ATCC 19213 with an exposure of up to 5 min at 25°C to 0.1% H_2O_2 and UV irradiation at an intensity of 0.8 mW/cm^2. The concentration of H_2O_2 had no effect on the viability over the short exposure. UV killing did occur with a *D*-value (time for killing of 90% of the population) of about 3 min, which equates to 1,483 J/m^2. Killing with the combined agents was biphasic in this experiment, with a *D*-value of about 0.67 min for the first phase and about 6 min for the second phase. Increasing the peroxide concentration did not increase synergy, and as Waites et al. *(16)* found, H_2O_2 had a shielding effect as well as a sensitizing effect. The synergy was specific for H_2O_2 and was not found for UV light and *t*-butyl hydroperoxide or peracetic acid *(12)*. However, this finding may be related to the use of UV light mainly at a wavelength of 254 nm, which is strongly absorbed by H_2O_2 but not by the other hydroperoxides. As expected, synergy required exposure to UV light and H_2O_2 at the same time with the liquid system used. Also, as found by other authors, synergy occurred only over a limited range of H_2O_2 concentrations—in our experiments, from about 0.05 to 0.40%.

The results of previous studies *(14, 15)* indicate that transition metal cations, particularly Fe^{2+} or Cu^+ but also the oxidized forms Fe^{3+} and Cu^{2+}, can protect spores against killing by H_2O_2. This same effect occurred in our studies with the combined UV-H_2O_2 treatment, as shown by the data presented in Figure 1B for Fe^{2+} and in Figure 1C for Cu^{2+}. The transition metal cations had essentially no effect on spore killing at 25°C by UV light alone (data not shown). The level of protection was dependent on the order of addition of the components to the mix. When $FeSO_4$ was added to H_2O_2 at the same time as or just before the addition to the spores, the salt was highly protective, but when it was added to the spores shortly after H_2O_2, the protective effect was greatly diminished or totally eliminated (data not shown). The results of previous studies *(12)* indicate that H_2O_2 is very rapidly taken up by spores. Apparently, the peroxide within spores does not react readily with Fe^{2+} added to the suspending medium. This effect may be the basis for the importance of order of addition in relation to UV-H_2O_2 spore killing. If Fe^{2+} or Cu^+ was added along with or before H_2O_2 but prior to irradiation (Fig. 1),



killing was reduced. If the cations were added to the spores after H_2O_2 and the spores were then UV irradiated, there was little reduction in killing. The simplest interpretation is that the transition metal cations rapidly catalyzed dismutation of H_2O_2 to oxygen and water outside the spores. This reaction can readily be demonstrated in terms of the visible production of gas (O_2) from mixtures containing solutions of $FeSO_4$ and H_2O_2 or the loss of H_2O_2 assayed with the horseradish-peroxidase method *(9)*.

For the experiments described in Figure 1, spore killing appeared to be predominantly by UV light, and 0.1% H_2O_2 alone had only a minimal effect at 25°C for the short times of exposure, although it clearly enhanced killing. However, at 50°C, H_2O_2 was more sporicidal, and, as shown by the data presented in Figure 2, it was possible to obtain synergistic killing under conditions in which H_2O_2 killing was greater than UV killing in control suspensions. Under these conditions, Fe^{2+} and Cu^{2+} were as highly protective against synergistic killing as they were at 25°C.

Thiosulfate at a concentration of 20 mM was at best only somewhat protective against UV-H_2O_2 killing at 25°C when UV damage was prominent, as shown by the example in Figure 3A, but was clearly more protective at 50°C when H_2O_2 was more damaging (Fig. 3B). Dimethylthiourea, a known scavenger of the hydroxyl radical, was also protective against UV-H_2O_2 killing, but the protection was approximately the same at 50°C as at 25°C. For example, an average 4-log reduction in viability was found for spores of *B. megaterium* at 25°C after exposure to UV light at a dose of 470 J/m^2 with 0.5% H_2O_2. An average of only a 1-log reduction was found for spores in the presence of 10 mM of dimethylthiourea. At 50°C, with a UV dose of 470 J/m^2 and 0.5% H_2O_2, there was a 5-log reduction in viability without dimethylthiourea but only a 1.5-log reduction with the scavenger present.

As shown in Figure 4A, 167 mM of pyruvate was protective against UV-H_2O_2 spore killing as well as for killing by UV light alone. As expected, pyruvate was protective against killing by H_2O_2 alone over a longer period of exposure at 25°C (Fig. 4B). Protection against peroxide killing presumably depends on previously documented reactions of H_2O_2 with pyruvate or other α-keto acids *(6)*. In our system with 0.1% (ca. 32.6 mM) H_2O_2, degradation at 25°C through reactions with 167 mM of pyruvate occurred at a rate of 0.78 μmol/ml/min when peroxide disappearance was assayed by the standard method involving the horseradish-peroxidase-leuco-crystal-violet procedure of Mottola

FIGURE 1. Killing of spores of B. megaterium *ATCC 19213 by peroxide and UV radiation. (A) Effects of +, 0.1% (ca. 32.6 mM) H_2O_2; □, UV irradiation; or a combination of the two (◇); (B) killing by UV light plus H_2O_2 of spores suspended in 0.1% H_2O_2 without (△) or with (▲) 10 mM of $FeSO_4$ added just prior to irradiation; (C) killing by UV light plus H_2O_2 of spores suspended in 0.1% H_2O_2 without (○) or with (●) 10 mM of $CuSO_4$ solution just prior to irradiation. One minute of irradiation corresponds to a UV dose of 480 J/m^2.*

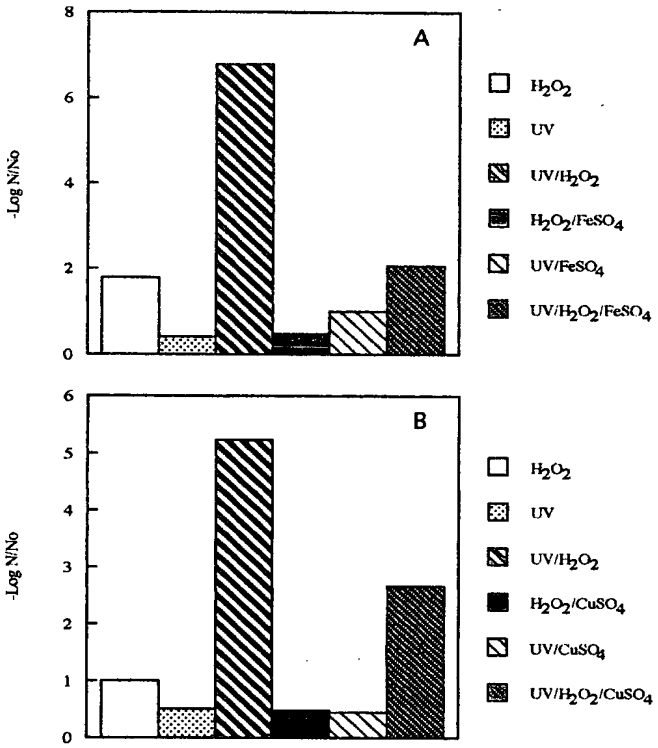


FIGURE 2. *Killing of spores of* B. megaterium *ATCC 19213 at 50°C by 0.15% H_2O_2 for 2 min and/or UV irradiation for 2 min at 3.5 μW/cm² for 2 min (4.2 J/m²) with or without 10 mM of $FeSO_4$ (A) or $CuSO_4$ (B).*

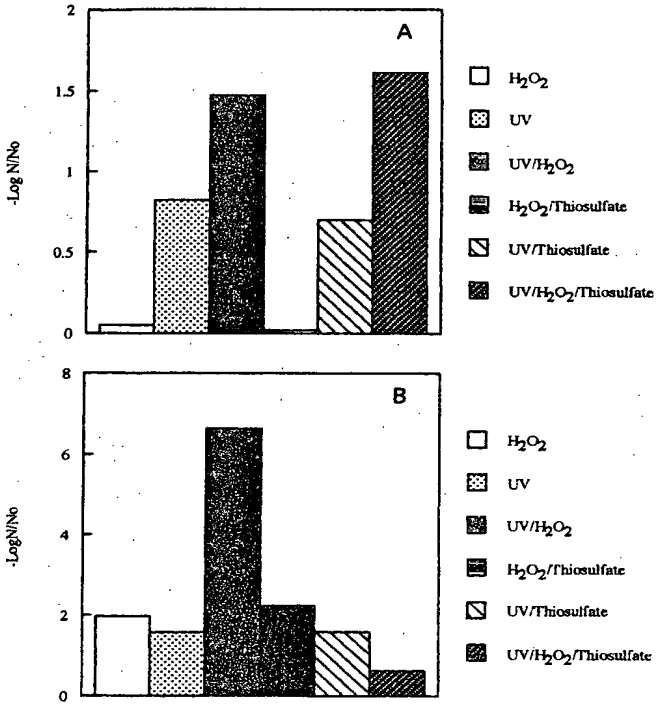


FIGURE 3. *Effects of 20 mM of sodium thiosulfate on killing of spores of* B. megaterium *ATCC 19213 at pH 7 and 25°C by UV irradiation at an intensity of 1.15 mW/cm² for 45 s (518 J/m²), by 0.1% H_2O_2 for 45 s, or by a combination of the two (A). Data are shown in (B) for the effects of 20 mM of sodium thiosulfate on killing at 50°C by 0.15% H_2O_2 for 2 min, UV irradiation at 3.5 μW·cm⁻² for 2 min (4.2 J/m²), or both.*

et al. *(9)*. This rate is somewhat lower than the rate of about 2.5 μmol/ml/min reported by Giandomenico et al. *(6)* for a mixture of 1 mM of pyruvate and 200 μM of H_2O_2 in water for 1 h at 37°C. The finding that pyruvate can protect against UV spore killing alone was surprising. Protection against UV killing presumably does not depend on peroxide degradation unless, of course, UV irradiation results in the formation of peroxides that can react with pyruvate. Still, pyruvate protection against both of the individual agents has to be considered in interpreting protection against the combined agents. At 50°C, pyruvate was highly protective against UV-H_2O_2 killing. For example, there was a 6.7-log reduction in the viable count of spores subjected to 0.15% H_2O_2 at 50°C for 2 min with UV irradiation at 3.5 μW/cm² (4.2 J/m²). Only a 0.3-log reduction in the viable count occurred when 167 mM of pyruvate was added to the spore suspension just prior to treatment with UV light and peroxide.

Use of UV irradiation at 222 nm. A shorter wavelength (222 nm) of UV irradiation proved to be more effective than the standard UV light from a mercury lamp (ca. wavelength of 254 nm) commonly used for spore killing. However, it was only somewhat more effective for killing spores either in aqueous suspensions or dried on glass, as shown by the sample data in Figure 5. Sample data are presented for *B. megaterium* spores dried on glass coverslips (Fig. 5A) and for *C. sporogenes* spores (Fig. 5B) in liquid suspensions.

As shown by the data in Figure 6, the shorter wavelength of UV light was effective for synergistic killing of spores of *B. subtilis* A, which is a spore type commonly used to assess the effectiveness of aseptic processing. The synergy for spores in suspensions is obvious from the data in Figure 6A. Also, we undertook experiments of the type described previously *(12)* for spores of *B. megaterium* or *C. sporogenes;* however, in our study, we used spores of *B. subtilis* A and with a UV wavelength of 222 nm. Spores of *B. subtilis* A were suspended in a solution containing 0.65% H_2O_2, and aliquots of the suspension were dried on glass coverslips at 25°C for various times before irradiation. Spores of *B. megaterium* and *C. sporogenes* can retain H_2O_2 under these drying conditions for as long as 24 h, although the peroxide completely evaporates from coverslips without spores *(12)*. The data presented in Figure 6B through D indicate that spores of *B. subtilis* A must also retain peroxide because, even after 6 or 12 h of drying, synergistic killing was evident. Even after 24 h of drying, the agents still appeared to have at least additive action. However, more prolonged drying resulted in further reductions in peroxide action. Similar results were obtained with spores of *B. megaterium* and *C. sporogenes* when UV light at wavelength 222 rather than 254 nm was used for the killing of cells previously exposed to H_2O_2 and then dried on coverslips for various times. Differences in the effectiveness of UV light at 222 versus 254 nm for synergistic

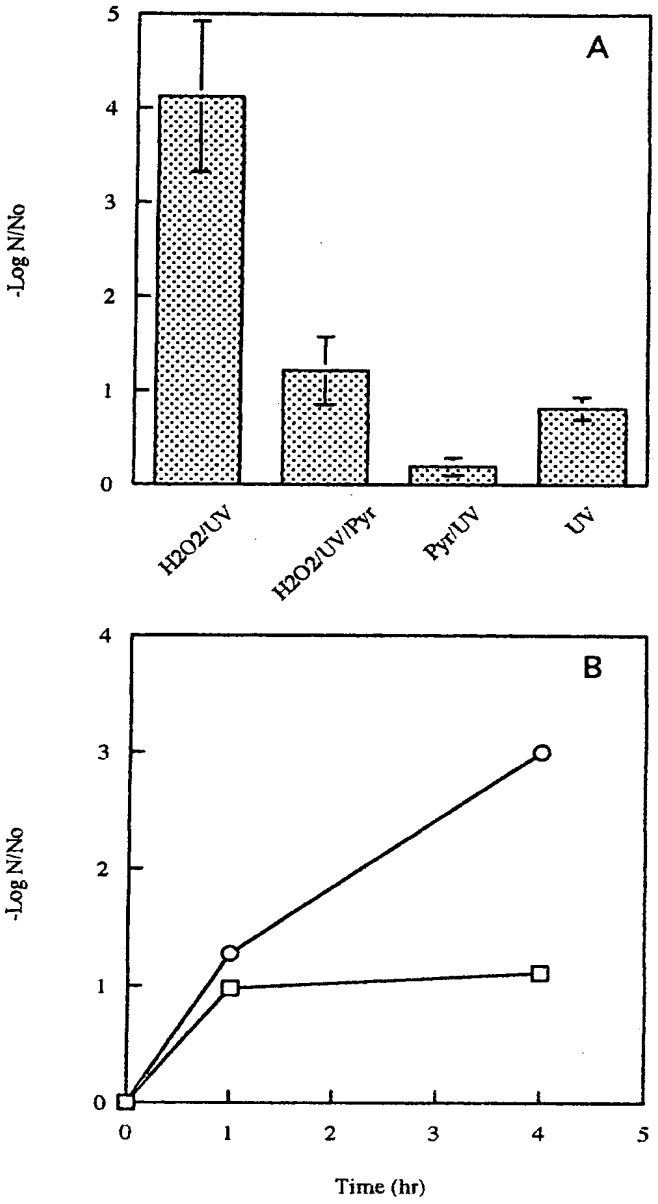


FIGURE 4. *Pyruvate inhibition of killing of spores of* B. megaterium *ATCC 19213. (A) Data for effects of 167 mM of sodium pyruvate on spore killing by UV irradiation at an intensity of 1.52 mW/cm² for 60 s (912 J/m²) at 25°C and of UV irradiation of spores in 0.1% H_2O_2 solution—bars show standard deviations with* n = 3*; (B) data that show inhibition by 167 mM of sodium pyruvate (□) on killing of spores by 0.5% H_2O_2 alone (○) at 25°C and pH 7 during a 4-h period.*

killing were too small for reliable distinctions to be made between the two (data not shown). However, UV light at 222 nm appears to be as effective as at 254 nm for synergistic killing.

Nature of the damage caused by UV light and H_2O_2. As shown by the data in Figure 7, auxotrophic mutants were obtained readily after dry heating. Spores of *B. megaterium* ATCC 19213, the strain used for most of our previous work on synergistic killing, were dried at room temperature on glass coverslips and then heated at 115°C for 5 min. About 2% of the survivors were able to grow in complex medium but not on defined Slepecky-Foster medium. The response to UV irradiation (270 J/m²) was less mutagenic, and fewer than 1% of the survivors were unable to grow on the defined medium. Combined UV-H_2O_2 treatment was even less mutagenic, while treatment with 0.1% H_2O_2 resulted in only very low levels of auxotrophic mutation. The treatment times varied from 5 min for dry heat to hours for H_2O_2 treatment at 25°C to achieve the desired levels of spore killing. Survivors were initially plated on Trypticase soy agar with incubation for at least 48 h to allow for the full growth of colonies. Then, colonies were picked randomly for testing. Dry-heat treatment was the positive control in the experiments. No treatment resulted in no detected auxotrophic mutations but, of course, also no killing. The differences among UV treatment, H_2O_2 treatment, and combined treatment were not statistically significant at the 95% confidence limit, although treatment with H_2O_2 alone appeared to be the least mutagenic. The conclusion is that combined UV-H_2O_2 treatment is not highly mutagenic for *B. megaterium* ATCC 19213 spores, at least not compared with the dry-heat treatment. The levels of auxotrophic mutation presented in this study for spores treated with peroxide are somewhat higher than those of, say, Fairhead et al. *(5),* but the organisms used were different, *B. subtilis* versus *B. megaterium.*

Results of our previous studies of spore killing by hydroperoxides and by heat have suggested that the inactivation of enzymes in the spore core may be involved in killing them *(11).* There is a wide spectrum of sensitivity among spore enzymes. For example, glucose 6-phosphate dehydrogenase in intact spores is highly sensitive to inactivation by H_2O_2, whereas F_1ATPase has very low sensitivity. As shown by the data in Figure 8, glucose 6-phosphate dehydrogenase was sensitive to inactivation by combined UV-H_2O_2 treatment. Under the experimental conditions, most of the inactivation appeared to be due to peroxide damage enhanced by the low level of UV irradiation used. However, glucose 6-phosphate dehydrogenase within spores could also be inactivated by UV irradiation alone.

DISCUSSION

The data presented suggest that the nature of combined UV-H_2O_2 killing can be affected by experimental conditions ranging from a process dominated by UV killing to one dominated by peroxide killing. In the sterilization of packaging materials, the killing is probably mainly dominated by peroxide because the materials are exposed to high concentrations of H_2O_2 at high temperatures before being UV irradiated. Thus, industrial conditions are closer in terms of temperature to our 50°C conditions under which thiosulfate is highly protective against killing. Of course, the industrial conditions involve much higher peroxide concentrations than we used and more rapid killing. Thus, our conditions only approach those of industrial units. Spores can take up very high concentrations of H_2O_2, even when exposed to fairly low concentrations of the agent *(12).*

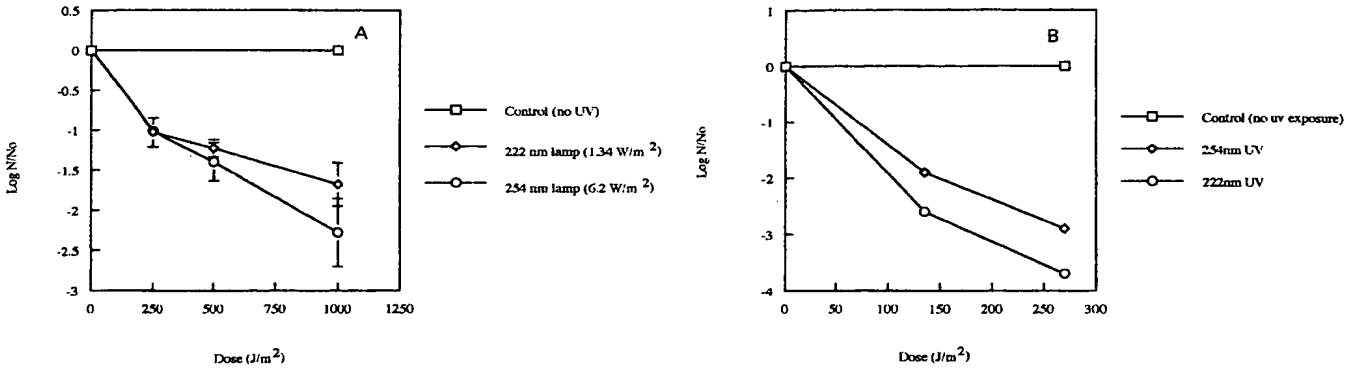


FIGURE 5. *Examples of relative sporicidal effectiveness of UV irradiation at 222 compared with UV irradiation at 254 nm for dried spores of* B. megaterium *(A) or for spores of* C. sporogenes *(B) in suspensions.*

Therefore, even if there is drying between peroxide exposure and UV irradiation, synergistic killing can still occur.

Fe or Cu cations or pyruvate are known protectors of spores against H_2O_2 damage, although Fe or Cu cations are well known to act oppositely to sensitize vegetative cells to peroxide killing. The findings presented indicate that they can also protect against combined UV-H_2O_2 spore killing. The agents appear to act primarily by chemically catalyzing H_2O_2 dismutation outside the spore or at least external to targets for damage.

UV light or H_2O_2, separately or in combination, did not appear to be highly mutagenic, in contrast to dry heat or ionizing radiation. The findings for the separate agents are consistent with those of other studies *(10, 13)*. These findings are generally explained as being the result of protection of the spore DNA against UV light or peroxide damage by small, acid-soluble spore proteins, with lesser contributions attributed to the physical state of the spore DNA and to DNA repair enzymes active after germination. It seems the same protective mechanisms apply to combined UV-H_2O_2 killing. Bayliss et al. *(1)* found that DNA repair-deficient spores had heightened sensitivities to the combined agents.

The finding that UV irradiation at 222 nm is as effec-

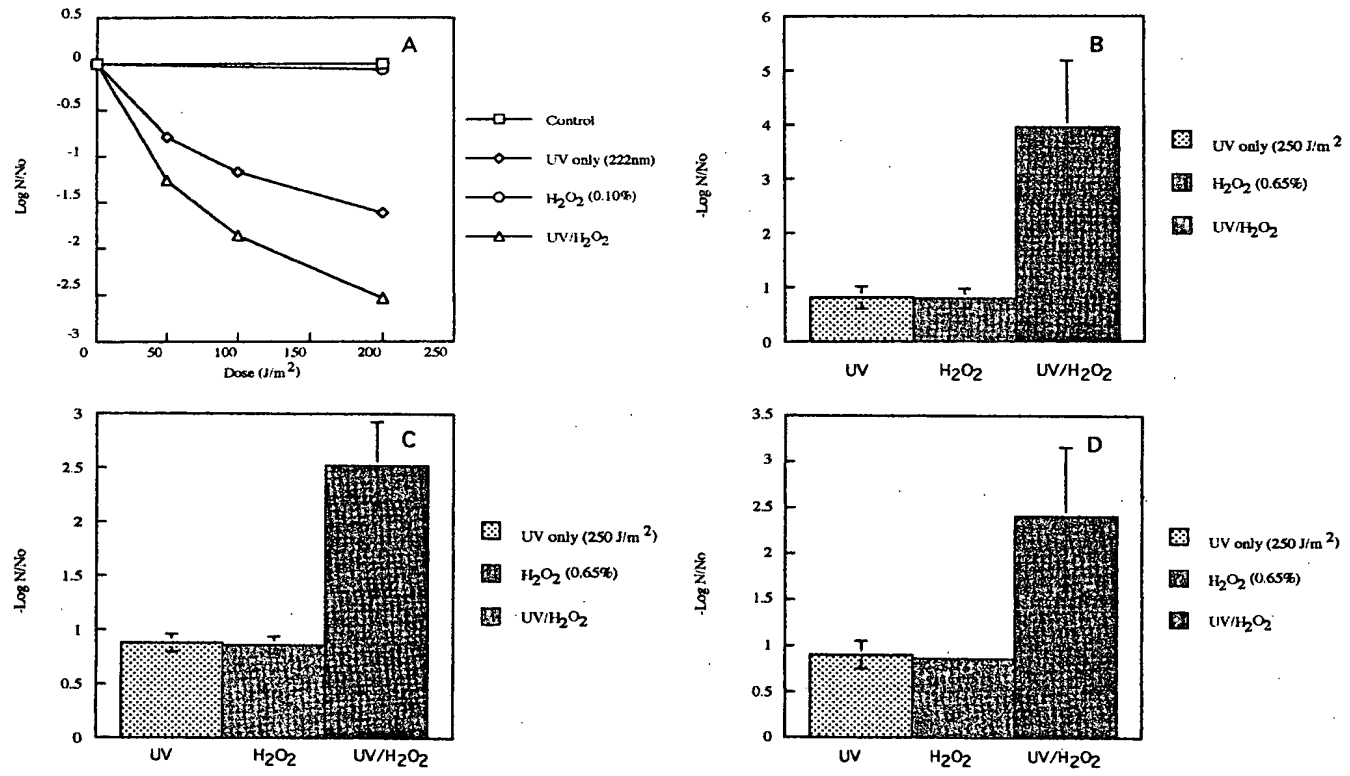


FIGURE 6. *(A) Synergistic killing of* B. subtilis *A spores suspended in 0.10% H_2O_2 solution at 25°C and then UV irradiated at 222 nm. Synergistic killing as assessed also for spores of* B. subtilis *A exposed initially to 0.65% H_2O_2 solution and then dried on glass coverslips at 25°C for 6 (B), 12 (C), or 24 (D) h before being UV irradiated at 222 nm.*

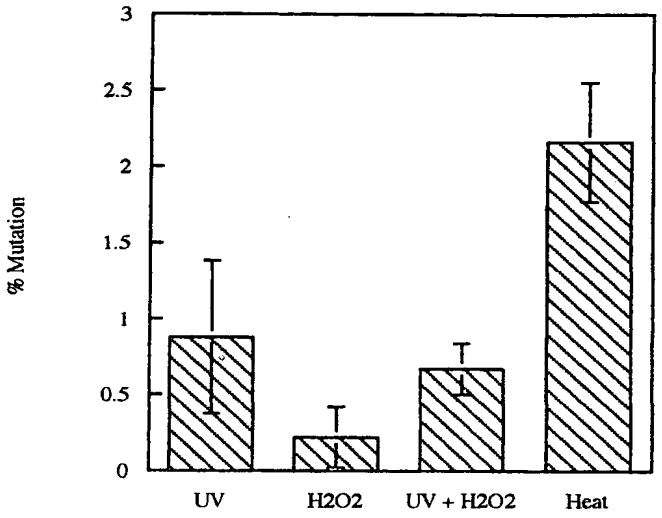


FIGURE 7. *Auxotrophic mutants among survivors of killing of spore populations by UV irradiation (270 J/m^2), 0.1% H_2O_2 at 25°C, pH 7, UV irradiation of spores in 0.1% H_2O_2 solution, or heating at 115°C of spores previously dried on glass coverslips. The error bars indicate standard deviations among multiple experiments with* n > 3.

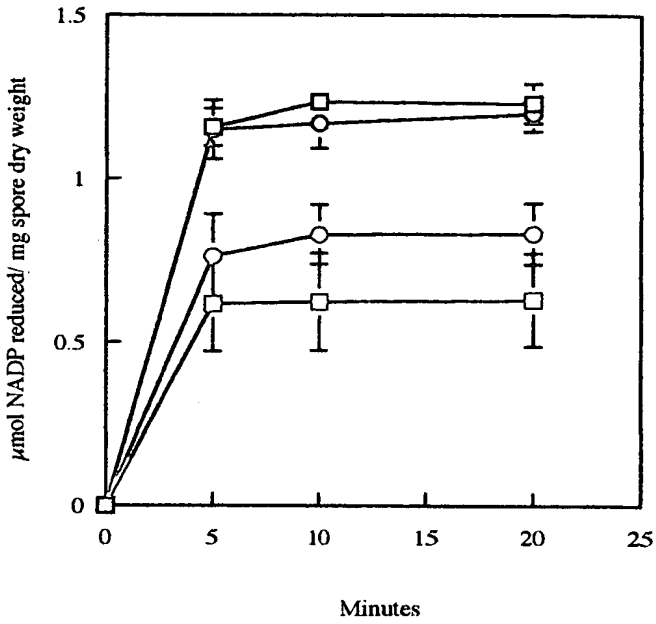


FIGURE 8. *Glucose 6-phosphate dehydrogenase activities of spores after 60 min of no treatment (□), UV treatment with 3.5 μW/cm^2 (126 J/m^2) (○), treatment with 0.1% H_2O_2 at 25°C, pH 7 (○), or treatment with both agents (□). Error bars indicate 95% confidence limits with three trials.*

tive as UV irradiation at 254 nm does not agree with previous findings *(16)* obtained with spores of *B. subtilis* NCDO 2129 suspended in 1% H_2O_2 solution and irradiated with UV light from wavelengths of 240 to 300 nm produced by a synchrotron radiation source. The basis for the difference is not clear, but it may be due to differences in experimental conditions. Also, in the previous work, the shortest wavelength used was 240 nm, and possibly, there is a trough in the curve relating sporicidal action to wavelength with a rise at 222 nm. Another possible difference is that we used more concentrated spore suspensions, about 10^9 versus about 10^7 spores per ml. However, as indicated above, in our experience, lowering the concentration of spores only somewhat enhances effectiveness of the agents but does not preclude synergy.

Our data indicate that proteins are targets for damage by the combined agents, as they are targets for damage by peroxides. We focused on glucose 6-phosphate dehydrogenase because of its known high sensitivity to peroxide damage *(11)*. Some spore enzymes are relatively insensitive to peroxide damage, e.g., F_1ATPase and pyruvate kinase. We found in separate experiments that both enzymes are also insensitive to combined peroxide-UV light damage, at least compared with glucose 6-phosphate dehydrogenase. The results of our findings with respect to the inactivation of glucose 6-phosphate dehydrogenase by the combined agents did not show major synergy, although inactivation by the combined agents was always greater than inactivation by any one of the agents. Overall, it seems that the inactivation of enzymes such as glucose 6-phosphate dehydrogenase by the combined agents could affect viability. However, cumulative damage to multiple enzyme targets is likely to be required for killing. In other words, there are multiple targets for the agents, and synergy is likely to arise from enhanced cumulative damage to multiple protein targets rather than to a single target.

Regardless of the targets for killing, it is apparent from this work as well as from previous studies *(12)* that synergistic killing occurs for a wide variety of spores and that exposure of spores to H_2O_2 can precede UV irradiation by many hours when spores are dried at room temperature or for shorter times when drying is at higher temperatures. UV irradiation at 222 nm was somewhat more effective for synergistic killing than UV irradiation at 254 nm, as it is for the killing of spores in suspensions or those dried on glass coverslips. The new findings should lead to more effective use of peroxide-UV light regimens for sterilization. Certainly, they allow more freedom in machine design.

REFERENCES

1. Bayliss, C. E., J. Shah, and W. M. Waites. 1982. Comparison of sensitivity of repair-proficient and repair-deficient strains of *Bacillus subtilis* to ultraviolet irradiation and hydrogen peroxide. *FEMS Microbiol. Lett.* 13:147–149.
2. Bayliss, C. E., and W. M. Waites. 1979. The synergistic killing of spores of *Bacillus subtilis* by hydrogen peroxide and ultra-violet light irradiation. *FEMS Microbiol. Lett.* 5:331–333.
3. Bayliss, C. E., and W. M. Waites. 1979. Combined effect of hydrogen peroxide and ultraviolet irradiation on bacterial spores. *J. Appl. Bacteriol.* 47:263–269.
4. Bayliss, C. E., and W. M. Waites. 1980. The effect of hydrogen peroxide and ultraviolet irradiation on non-sporing bacteria. *J. Appl. Bacteriol.* 48:417–422.
5. Fairhead, H., B. Setlow, and P. Setlow. 1993. Prevention of DNA damage in spores and *in vitro* by small, acid-soluble proteins from *Bacillus* species. *J. Bacteriol.* 175:1367–1374.
6. Giandomenico, A. R., C. E. Cerniglia, J. E. Biaglow, C. W. Stevens, and C. J. Koch. 1997 The importance of sodium pyruvate in assess-

ing damage produced by hydrogen peroxide. *Free Rad. Biol. Med.* 23:426–434.
7. Kim, J., and B. Naylor. 1966. Spore production by *Bacillus stearothermophilus*. *Appl. Microbiol.* 14:690–691.
8. Marquis, R. E., G. C. Rutherford, M. M. Faraci, and S.-Y. Shin. 1995. Sporicidal action of peracetic acid and protective effects of transition metal ions. *J. Ind. Microbiol.* 15:486–492.
9. Mottola, H. A., B. E. Simpson, and G. Gorin. 1970. Absorptiometric determination of hydrogen peroxide in submicrogram amounts with leuco crystal violet and peroxidase as catalyst. *Anal. Chem.* 42:410–411.
10. Nicholson, W. L., N. Munakata, G. Horneck, H. J. Melosh, and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64:548–572.
11. Palop, A., G. C. Rutherford, and R. E. Marquis. 1998. Inactivation of enzymes within spores of *Bacillus megaterium* ATCC 19213 by hydroperoxides. *Can. J. Microbiol.* 44:465–470.
12. Rutherford, G. C., J. S. Reidmiller, and R. E. Marquis. 2000. Method to sensitize bacterial spores to subsequent killing by dry heat or ultraviolet irradiation. *J. Microbiol. Methods* 42:281–290.
13. Setlow, P. 1995. Mechanisms for the prevention of damage to DNA in spores of *Bacillus* species. *Annu. Rev. Microbiol.* 49:29–54.
14. Shin, S.-Y., E. G. Calvisi, T. C. Beaman, H. S. Pankratz, P. Gerhardt, and R. E. Marquis. 1994. Microscopic and thermal characterization of hydrogen peroxide killing and lysis of spores and protection by transition metal ions, chelators, and antioxidants. *Appl. Environ. Microbiol.* 60:3192–3197.
15. Waites, W. M., C. E. Bayliss, N. R. King, and A. M. C. Davies. 1979. The effect of transition metal ions on the resistance of bacterial spores to hydrogen peroxide and heat. *J. Gen. Microbiol.* 112:225–233.
16. Waites, W. M., S. E. Harding, D. R. Fowler, S. H. Jones, D. Shaw, and M. Martin. 1988. The destruction of spores of *Bacillus subtilis* by the combined effects of hydrogen peroxide and ultraviolet light. *Lett. Appl. Microbiol.* 7:39–140.